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N-Acetylglucosaminyl Transferases From the Pupal Instar of the Stable Fly, *Stomoxys calcitrans*

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N-acetylglucosaminyl transferases from pupae of *Stomoxys calcitrans* (L.) were studied in 10,000*g* pellet suspensions. Characterization of these enzymes was based on formation of glycolipids (ie, Dol·PP-GlcNAc and Dol·PP-$(GlcNAc)_2$), oligosaccharide lipids, and glycoproteins. Studies on transferase activity during the pupal instar showed that there were two peaks of activity; the first peak was on day 0 (prepupae) and the second at 3 days after pupation. Subcellular fractionation indicated that 10,000*g* and 100,000*g* pellets contained most of the transferase activities. The transferases required divalent cations (either Mn^{2+} or Mg^{2+}). The pH optimum, which varied for each of the products formed, was 7.5 for glycolipids, 7.0 for oligosaccharide lipids, and 6.5 for glycoprotein. Inclusion of dolichol monophosphate doubled the amount of Dol·PP-GlcNAc and Dol·PP·$(GlcNAc)_2$ formed, but had little effect on oligosaccharide lipid and glycoprotein formation. Tunicamycin was a potent inhibitor of glycolipid formation with an I_{50} of 1.8–4.8 nM. It was confirmed that tunicamycin acts by preventing the transfer of GlcNAc-1-P from UDP-GlcNAc to Dol·P. UMP reverses glycolipid formation, yielding UDP-GlcNAc. Some characterization of the products was performed. Glycolipids were shown to be Dol·PP-GlcNAc and Dol·PP-$(GlcNAc)_2$. Glycoprotein was rapidly solubilized by protease and detergent treatments, whereas oligosaccharide lipids appeared to be acid-labile, pyrophosphate-containing lipids. The apparent kinetic constants for the formation of glycolipids were as follows: UDP-GlcNAc $K_m = 1.55 \pm 0.47\ \mu M$, $V_{max} = 0.66 \pm 0.21$ pmol·min^{-1}·mg^{-1}; Dol·P $K_m = 2.08 \pm 0.85\ \mu M$, $V_{max} = 0.13 \pm 0.06$ pmol·min^{-1}·mg^{-1} protein.

Key words: stable fly, ***Stomoxys calcitrans,*** N-acetylglucosaminyl transferases, glycosyltransferases, tunicamycin, carbohydrate metabolism

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INTRODUCTION

Glycosyl transferases are heavily involved in glycoprotein synthesis and are ubiquitous in nature [1,2]. These enzymes transfer carbohydrates from sugar nucleotides directly to proteins or indirectly through lipid intermediates. Dolichol phosphate or a similar lipid often acts as the acceptor molecule for the indirect transfer of carbohydrates to proteins [1,2]. A commonly accepted pathway [2] for the biosynthesis of oligosaccharides via Dol·P* and their subsequent en bloc transfer to proteins is given in Figure 1. Evidence that such a pathway exists in insects has been reported [3–7].

Carbohydrate metabolism involving chitin metabolism in insects has been under investigation in our laboratory for the past few years [8–10]. Recently we have focused our attention on glycosyl transferases because they may be involved in chitin biosynthesis [11] or they may be the site of action for certain chitin synthesis inhibitors. Indirect proof of their involvement comes from the fact that the antibiotic tunicamycin has been reported to inhibit chitin synthesis in vitro [12]. Tunicamycin has been shown to inhibit specifically the transfer of GlcNAc-1-P from UDP-GlcNAc to Dol·P (refer to Fig. 1), thereby inhibiting oligosaccharide lipid and glycoprotein synthesis [13,14]. If tunicamycin does act in this way in insect tissues, then the observed inhibition of chitin synthesis could result from competition between UDP-GlcNAc and tunicamycin for active sites on chitin synthase [12], or it may result from inhibiting the synthesis of GlcNAc-labeled oligosaccharides which may act as primers for chitin biosynthesis [11].

The present report describes GlcNAc transferases isolated from stable fly *(Stomoxys calcitrans L.)* pupae. Some characterization of the various products is given (ie, glycolipids, oligosaccharide lipids, and glycoproteins). The specific site of action for tunicamycin in insect tissues is reported.

MATERIALS AND METHODS

Chemicals

UDP-[glucosamine-6-^{3}H] GlcNAc (24 Ci/mmole) and UDP-[glucosamine-1-^{14}C] GlcNAc (35 mCi/mmole) were purchased, respectively, from New England Nuclear, Boston, and ICN Pharmaceuticals, Irvine, CA. Betafluor and Bray's scintillation cocktails were purchased from National Diagnostics, Somerville, NJ. Dolichol monophosphate (80–90% pure), dithiothreitol, nonradioactive carbohydrates, and MOPS and MES buffers were purchased from Sigma Chemical Co, St. Louis, MO. Diflubenzuron (Dimilin; TH-6040; N-(((4-chlorophenyl)amino)carbonyl)-2,6-diflubenzamide) was a gift from Du-

*Abbrevations: dolichol monophosphate = Dol·P; dolichol pyrophosphate = Dol·PP; 3-(N-morpholino)-propanesulfonic acid = MOPS; dithiothreitol = DTT

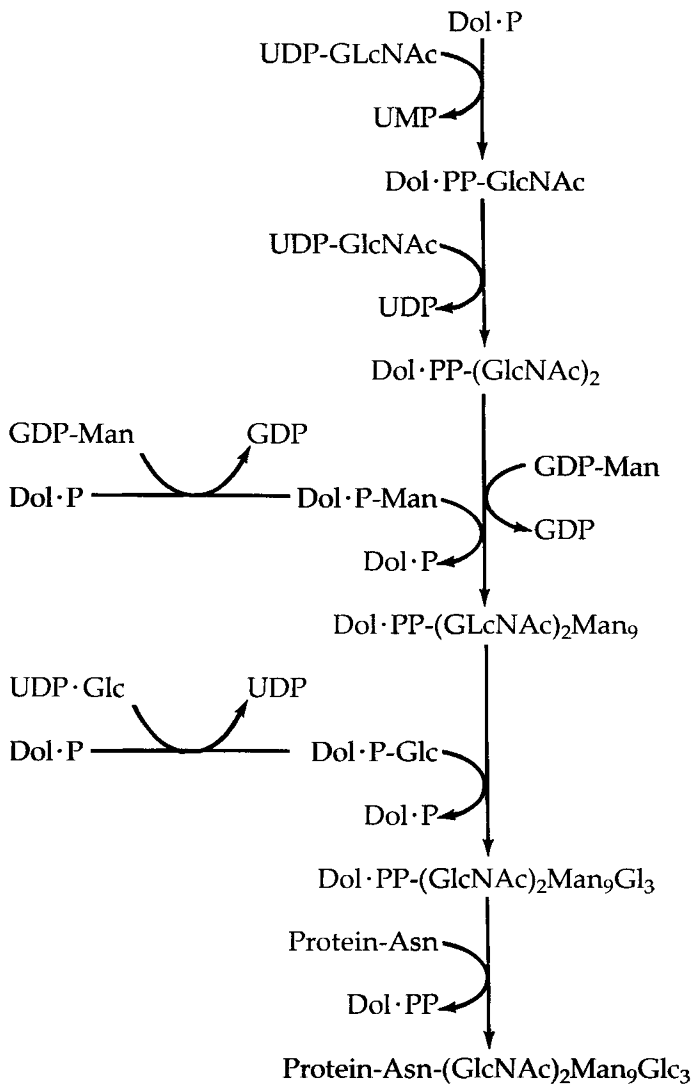

Fig. 1. Flow chart of glycoprotein synthesis and the inolvement of glycosyl transferases [2].

phar, B.V., Holland. Tunicamycin was provided by Dr T.D. Douros, Developmental Therapeutics Program, Chemotherapy, NCI, Bethesda, MD.

Insects

Stable flies were obtained as white prepupae from cultures maintained in this laboratory [15]. Prior to homogenization the prepupae were surface-disinfected with 70% ethanol [8]. For some experiments the prepupae were transferred to Petri dishes and incubated at 27°C for up to 7 days.

Enzyme Preparation

Stable fly prepupae or pupae were homogenized with a Polytron homogenizer in four volumes of cold 50 mM MOPS, pH 7.5, containing 0.25 M sucrose, 1 mM EDTA, and 2.5 mM DTT. The homogenate was filtered through four layers of cheesecloth, then subjected to a differential centrifugation scheme as described previously [9]. Final resuspension of pellets (10,000*g* or 100,000*g*) was in 50 mM MOPS, pH 7.5, containing 2.5 mM DTT and 2 mM $MgCl_2$.

Assay of GlcNAc Transferases

The reaction mixtures (400 μl total volume) generally contained 40 μg Dol·P, 0.1% (final) Triton X-100, 20 μmol MOPS (pH 7.5) containing 0.8 μmol $MgCl_2$, 1 μmol DTT, and 100 μl enzyme (ca 1.5 mg protein). When there were other additives the buffer was adjusted so that the final volume was 0.4 ml. Dol·P was suspended by sonicating the reaction mixture for 5 (100 w) before adding the enzyme, using a Braun-sonic sonicator equipped with a microprobe. Inhibitors were added 5 min prior to initiation of the reaction with either [^{3}H](125,000–180,000 dpm)- or [^{14}C](175,000 dpm)-labeled UDP-GlcNAc. The radiolabeled substrate was first dried to remove ethanol, then dissolved in buffer before it was added to the reaction mixture. Reactions were normally conducted at 30°C for 15 min. The reactions were terminated by addition of 4 ml $CHCl_3/CH_3OH$ (1/1) and 1.4 ml H_2O. Subsequent fractionation of the products into glycolipid (ie, Dol·P-GlcNAc and Dol·PP-$(GlcNAc)_2$), oligosaccharide lipids, and glycoprotein was according to Forsee and Elbein [16]. Because the glycolipid fraction (ie, $CHCl_3/CH_3OH$-soluble material) contained a number of neutral lipid products that obscured the analysis of Dol·PP-GlcNAc and Dol·PP-$(GlcNAc)_2$, this fraction was subjected to a cleanup procedure as follows. The glycolipid fractions were added to test tubes containing 200 mg (dry weight) of DEAE-cellulose that had been previously washed once with 6 ml $CHCl_3/CH_3OH$ (2/1), twice with 4 ml 0.1 M ammonium acetate in CH_3OH, three times with 4 ml CH_3OH, and twice with 4 ml $CHCl_3/CH_3OH$ (2/1). The $CHCl_3$ phases were vortexed with the DEAE cellulose, then centrifuged at 2,000*g* for 2 min. Afterward, the supernatant was removed by aspiration. The DEAE cellulose was then sequentially washed four times with 4 ml (each wash) $CHCl_3/CH_3OH$ (2/1) and four times with 4 ml (each wash) CH_3OH. The DEAE-cellulose was vortexed after each solvent addition and centrifuged as before; then the supernatant was removed by aspiration. The Dol·PP-GlcNAc and Dol·PP-$(GlcNAc)_2$ were then eluted from the DEAE-cellulose by washing three times with 4 ml (each wash) 0.1 M ammonium acetate in CH_3OH. The supernatants (washes) were removed and added directly to scintillation vials or pooled for analysis by TLC or paper chromatography. With these latter samples, the solvent was removed by evaporation and then redissolved in 40 ml $CHCl_3/CH_3OH$ (2/1). Ammonium acetate was removed by washing with 16 ml H_2O. The $CHCl_3$ layer was removed and evaporated; the residue was again redissolved in 40 ml $CHCl_3/CH_3OH$ and washed a second time with 16 ml H_2O. The $CHCl_3$ layer containing the Dol·PP-GlcNAc and Dol·PP-$(GlcNAc)_2$ was then ready for further analysis.

Chromatography

The N-acetylglucosaminyl lipids were analyzed by chromatography on 20 × 20 cm TLC plates coated with 0.25 mm silica gel G (Analtech, Newark, DE). The TLC plates were developed in closed chambers with wicks in the following solvent system: Solvent A—$CHCl_3/CH_3OH/H_2O$ (65/25/4).

Nucleotides, carbohydrates, and oligosaccharides were analyzed by paper chromatography on Whatman No. 1 paper. Development of the chromatograms was in one of the following solvent systems: Solvent B—n-butanol/pyridine/H_2O (6/4/3); Solvent C—ethanol/1 M ammonium acetate, pH 7.4 (7/3); Solvent D—isobutyric acid/concentrated NH_4OH/H_2O (57/4/39. Radioactive areas were located by cutting the chromatograms into 1-cm segments, placing the segments in scintillation vials containing Betafluor scintillation cocktail, then measuring the radioactivity in a scintillation spectrometer. Carbohydrate standards were visualized with alkaline silver nitrate [17].

Purification and Characterization of N-Acetylglucosaminyl Lipids

Glycolipids were isolated by solvent extraction as described above and then were purified on a DEAE-cellulose column following the procedure of Scher et al [18]. Fractions (ca 8 ml) were collected and the radioactivity measured. Tubes containing radioactivity that were collected during the application of ammonium acetate were pooled, dried, and redissolved in $CHCl_3/CH_3OH$ (2/1, 40 ml). Ammonium acetate was removed by washing the sample with 16 ml of H_2O in a separatory funnel; this step was repeated twice. The organic layer was dried, dissolved in 1–2 ml of $CHCl_3/CH_3OH$ (2/1), and applied to TLC plates for analysis. In some instances the lipids were hydrolyzed in 0.01 N HCl in 50% n-propanol at 100°C for 10 min. The samples were neutralized with Na_2CO_3 after hydrolysis, then extracted with $CHCl_3/CH_3OH$ (2/1) to remove the lipids. The aqueous layer was removed, dried, redissolved in H_2O, and spotted on Whatman No. 1 paper. Paper chromatograms were developed in Solvent B. Standards of GlcNAc and $(GlcNAc)_2$ were chromatographed along with the samples. Radioactivity was located by cutting the paper into 1-cm sections, placing the sections in scintillation vials filled with Bray's scintillation cocktail, and measuring the radioactivity in a scintillation spectrometer.

Characterization of Oligosaccharide Lipids

Oligosaccharide lipids (10/10/3, $CHCl_3/CH_3OH/H_2O$-soluble) were isolated by solvent extraction and chromatographed on Whatman No. 1 paper developed with either Solvent D or distilled H_2O.

Proteolytic and Detergent Solubilization of Glycoprotein

Glycoprotein was subjected to proteolytic digestion by protease (pronase) (type VIII, Sigma Chemical Co, St Louis). Glycoprotein was suspended in 2–4 ml of 20 mM Tris buffer, pH 7.9, containing 10 mM $CaCl_2$ and 1 mg protease per milliliter. The mixture was incubated at 30°C in a shaking water bath for 24 h. Afterward, the soluble and insoluble materials were separated by centrifugation. The insoluble material was resuspended, and aliquots were removed from the soluble and insoluble fractions for measurement of radioactivity.

In addition, glycoprotein was suspended in 1% (w/v) sodium dodecyl sulfate and allowed to stand for 30 min at room temperature. The suspension was centrifuged at 2,000*g* for 15 min to isolate insoluble protein. The supernatant was removed and the pellet resuspended in 1 ml H_2O. Aliquots were placed in scintillation vials containing Bray's scintillation cocktail, and the radioactivity was measured with a scintillation spectrometer.

Reversal of the Transferase Reaction

Experiments were conducted in the following manner to determine if the GlcNAc transferase reaction could be reversed. The reaction was increased (5-fold) and allowed to continue in the usual manner for 10 min. The reaction tubes were then put in ice and subsequently centrifuged at 10,000g for 15 min at 4°C. The supernatant was aspirated off and the pellet resuspended in 1.8 ml of ice-cold reaction buffer. Nothing was added to the controls, but 5 mM either UMP or UDP was added to the test samples. The 0-min control reaction was stopped immediately after it was returned to the water-bath shaker, by addition of 16 ml $CHCl_3/CH_3OH$ (1/1, v/v) and 7 ml H_2O. The other reactions were allowed to continue 5 min and then stopped. The $CHCl_3$ layer was removed and saved. The H_2O layer was washed once more with 8 ml $CHCl_3$. The $CHCl_3$ layer was removed, pooled with the first, and processed using DEAE-cellulose as described above. The H_2O layers were freeze-dried and redissolved in 200 μl H_2O. Aliquots (20 μl) were applied to Whatman No. 1 paper and developed in Solvent C. After development, the chromatogram was cut into 1-cm segments which were put into scintillation vials containing Betafluor scintillation cocktail. GlcNAc and UDP-GLcNAc were identified by chromatographing known standards along with the samples.

Protein and Phosphorus

Protein concentration was determined by the method of Bradford [19]. Phosphorus was determined according to Duck-Chong [20].

Kinetic Constants for Glycolipid Formation

Apparent kinetic constants were determined by considering Dol·PP-GlcNAc and Dol·PP-$(GlcNAc)_2$ as one product. Kinetic experiments were conducted as described above, except that the reaction time was limited to 10 min and the concentration of either UDP-GlcNAc or Dol·P was varied. Dol·P concentrations were based on P content, and varied from 1.5 to 60 μM while holding UDP-GlcNAc constant at 120 μM. UDP-GlcNAc concentrations were varied from 62.5 nM to 2.62 μM while holding Dol·P constant at 60 μM.

Statistical Treatment of Data

Data presented in the figures and tables are the means $\pm$ SD.

RESULTS

Subcellular Distribution

Initially, we performed experiments to determine which subcellular fraction possessed the greatest GlcNAc transferase activity (Table 1). The 10,000*g* pellet mitochondrial fraction had the highest overall incorporation for the products we examined. The 100,000*g* pellet microsomal fraction had a higher activity for the formation of glycolipid. The mitochondrial fraction had appreciable incorporation of all three products and was therefore chosen as our enzyme source.

Effects of K^+ and Mg^{2+} Cations

Monovalent ions such as K^+ stimulated glycolipid and oligosaccharide lipid formation maximally at 20–40 mM, but activity was depressed at higher concentrations. Transfer of GlcNAc to protein was inhibited at all concentrations of K^+ examined.

Magnesium ions also stimulated transfer of GlcNAc to the various products (Fig. 2). Magnesium ion stimulated production of glycolipid at 2.5 mM Mg^{2+}. GlcNAc transfer to oligosaccharide lipids and glycoprotein was maximally stimulated at 0.1 mM Mg^{2+}, and then decreased sharply to 1 mM Mg^{2+}, where activity appeared to plateau.

pH Effects

The pH of the reaction mixture affected the formation of glycoprotein, oligosaccharide lipids, and glycolipids differently. Optimum pH's for the formation of oligosaccharide lipids, glycoprotein, and glycolipids were 7.0, 6.5, and 7.5, respectively. We optimized conditions at pH 7.5 for glycolipid formation because 60–70% of the substrate ended up in neutral lipids.

Effect of Dolicholmonophosphate

It is well established that carbohydrate portions of glycoproteins are synthesized by membrane-bound enzymes as complex oligosaccharyldiphosphoryl dolichols, which are subsequently transferred to asparagine residues of proteins [1,2]. Previous studies have shown that insects are capable of transferring GlcNAc units from UDP-GlcNAc to Dol·P [4].

TABLE 1. Typical Subcellular Distribution Study of N-Acetylglucosaminyl Transferases in 0-Day Pupae of the Stable Fly

	GL[a] (dpm/mg)	Total		OL[a] (dpm/mg)	Total		GP[a] (dpm/mg)	Total	
		dpm	%		dpm	%		dpm	%
Homogenate[b]	568	1,448,035	100	87	223,125	100	75	191,250	100
1,000g pellet	1,776	599,487	41.4	150	50,608	22.7	764	257,869	134.8
10,000g pellet	2,330	131,126	9.1	651	38,911	17.4	936	55,860	29.2
100,000g pellet	3,364	191,426	13.2	167	9,550	4.3	75	4,266	2.2
100,000g supernatant	46	68,946	4.8	5	7,955	3.6	82	121,982	63.8

[a]GL = glycolipids, OL = oligosaccharide lipids, GP = glycoproteins.
[b]There were 1.11×10^7 dpm per reaction.

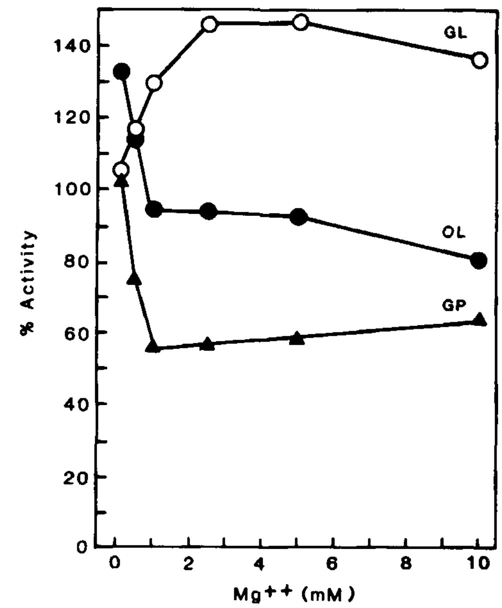

Fig. 2. Effect of Mg^{2+} on N-acetylglucosaminyl transferase activity in 10,000g pellet suspensions from 0-day stable fly pupae. Reactions were conducted as described in Methods, except Mg^{2+} concentration was varied. ○ = GL, glycolipids; ● = OL, oligosaccharide lipids; ▲- GP, glycoproteins.

TABLE 2. Effects of Dol·P on GlcNAc Transferases

	DPM		
Dol·P	GL[a]	OL[a]	GP[a]
0	3,346 ± 19	2,241 ± 30	6,403 ± 1,078
30 μM	8,586 ± 123	2,526 ± 366	5,676 ± 769

[a]GL = glycolipids; OL = oligosaccharide lipids, GP = glycoproteins.

We therefore wanted to determine the effect of adding exogenous Dol·P to the reaction mixtures. The inclusion of Dol·P in the GlcNAc transferase reaction mixtures more than doubled the amount of glycolipids formed (Table 2). We tried several concentrations of Dol·P, and 30 μM was found to be optimal for glycolipid formation at these conditions. At this concentration of Dol·P (30 μM), there was no effect on the formation of oligosaccharide lipids or glycoprotein. Higher concentrations (eg, 60 μM) inhibited glycoprotein production (data not shown).

Time Course of GlcNAc Transferase

The incorporation rate of GlcNAc into glycolipids, oligosaccharide lipids, and glycoprotein varied. Figure 3 shows incorporation of GlcNAc into these products over a 60-min time span. Incorporation of GlcNAc into glycolipids is linear up to 30 min and then declines. Oligosaccharide lipid formation did

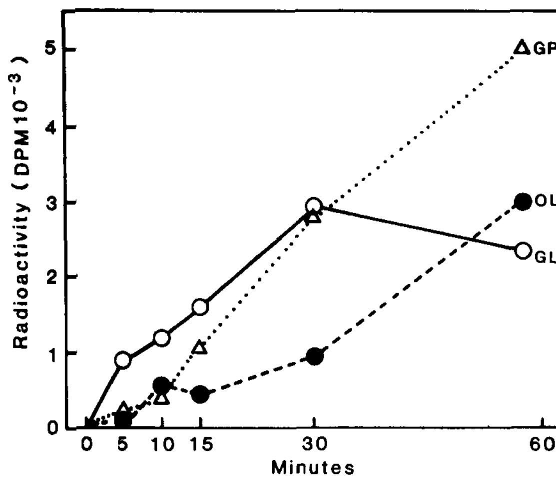

Fig. 3. Time course for the N-acetylglucosaminyl transferase reactions obtained with 10,000*g* pellet suspension from 0-day stable fly pupae. Reactions were conducted as described in Methods. ○ = GL, glycolipids; ● = OL, oligosaccharide lipids; △ = GP, glycoproteins.

not appear to be linear. Incorporation of GlcNAc into glycoprotein lags 5–10 min, then becomes relatively linear to 60 min.

N-Acetylglucosaminyl Transferase Activity in the Pupal Instar

It was of interest to follow N-acetylglucosaminyl transferase activities during the pupal instar of the stable fly to determine where the highest activities were. Glycolipid production through the pupal instar is shown in Figure 4. The highest activity occurred on day 0-ie, with prepupae. A small rise in activity occurred on day 3; then activity steadily declined until adult emergence. Oligosaccharide and glycoprotein production followed the same general trend.

Effect of Chemicals on GlcNAc Incorporation

Eight substances were tested to determine their effects on GlcNAc incorporation. The results are shown in Table 3. One nucleoside tested, uridine, inhibited all product groups at the 50 and 100 μM levels. The nucleotides UMP, UDP, and UTP also exhibited inhibitory effects. UTP was not as good an inhibitor as UMP and UDP for glycolipid formation, but the levels of inhibition of oligosaccharide and glycoprotein were almost the same for the three nucleotides.

In several reports [5,21,22], the presence of GDP-Man was necessary for the synthesis of GlcNAc-labeled oligosaccharides. The results presented in Table 3 show that GDP-Man had no significant effect on the formation of the three products. This is discussed further below.

As expected, EDTA inhibited product formation. This is probably due to divalent cations being chelated by EDTA.

Polyoxin D and tunicamycin were tested for inhibitory effects. Although polyoxins are structurally similar to UDP-GlcNAc [23] and have been re-

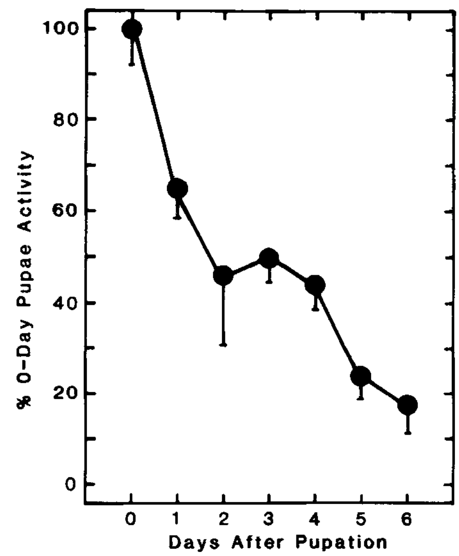

Fig. 4. Temporal study of N-acetylglucosaminyl transferase acvtivity for formation of glycolipids. Conditions as described in Methods.

TABLE 3. Effects of Chemicals on GlcNAc Incorporation

		Percent control		
		GL[a]	OL[a]	GP[a]
Uridine	10 μM	105.3 ± 8.0	87.9 ± 11.8	88.6 ± 27.6
	50 μM	88.2 ± 6.5	74.2 ± 5.0	56.7 ± 6.3
	100 μM	68.3 ± 3.5	70.1 ± 11.2	53.2 ± 10.7
UMP	10 μM	92.4 ± 8.1	77.1 ± 27.1	82.8 ± 8.9
	50 μM	61.3 ± 7.4	64.6 ± 18.7	66.7 ± 9.6
	100 μM	55.9 ± 1.2	33.3 ± 10.4	47.3 ± 2.1
UDP	10 μM	91.6 ± 6.9	66.7 ± 22.9	86.0 ± 9.3
	50 μM	72.7 ± 4.1	47.9 ± 10.4	73.5 ± 20.8
	100 μM	63.2 ± 9.5	52.1 ± 22.4	42.6 ± 5.4
UTP	10 μM	100.5 ± 2.6	76.1 ± 12.9	66.9 ± 26.4
	50 μM	90.7 ± 0.8	56.9 ± 12.8	39.9 ± 9.4
	100 μM	81.5 ± 1.9	46.9 ± 5.0	43.8 ± 8.4
GDP-MAN	50 μM	87.1 ± 25.3	80.3 ± 2.5	110.4 ± 9.7
	100 μM	72.8 ± 26.9	86.3 ± 2.4	92.2 ± 3.9
	400 μM	83.1 ± 1.8	87.5 ± 19.8	93.2 ± 6.5
EDTA	25 μM	21.1 ± 5.8	55.4 ± 11.4	52.7 ± 19.7
Polyoxin	1 μM	110.1 ± 10.6	101.1 ± 6.8	113.2 ± 16.2
	5 μM	97.7 ± 6.2	87.5 ± 12.9	89.4 ± 13.0
	10 μM	107.5 ± 0.5	105.7 ± 6.1	99.2 ± 12.0
Tunicamycin	0.6 μM	37.8 ± 3.8	111.4 ± 7.0	82.7 ± 13.8

[a]GL = glycolipids; OL = oligosaccharide lipids; GP = glycoproteins.

ported to inhibit other UDP-GlcNAc-requiring enzymes such as chitin synthase [9,24], polyoxin D did not inhibit the GlcNAc transferase studied here. Tunicamycin, which is an antibiotic containing glucosamine, was found to be a potent inhibitor of glycolipid formation but not of oligosaccharide lipid or glycoprotein formation in this in vitro assay system. Complete inhibition of glycolipid formation was not observed; maximal inhibition was 65–70%. Inhibition of glycolipid formation, but not oligosaccharide lipids or glycoproteins, could be explained by the fact that tunicamycin has been shown to inhibit the addition of GlcNAc-1-P from UDP-GlcNAc to a lipid acceptor (ie, Dol·P) in other organisms [13,14]. Dol·P-GlcNAc and Dol·P-$(GlcNAc)_2$ are in turn further glycosylated (oligosaccharide lipids; refer to Fig. 1); the oligosaccharides are then transferred in their entirety to protein acceptors. The presence of endogenous glycolipids or oligosaccharide lipids that could be further glycosylated and finally transferred to proteins would explain the lack of an inhibitory effect on glycoprotein and oligosaccharide lipid formation and the fact that complete inhibition of glycolipids was not observed. The I_{50} for glycolipid formation by tunicamycin was graphically determined to be 1.8–4.8 nM, assuming a molecular weight of 830.9 [25].

We further investigated the mode of action of tunicamycin to confirm that it inhibited the transfer of GlcNAc-1-P to Dol·P, but not the transfer of the second GlcNAc. To determine this, we prepared unlabeled Dol·PP-GlcNAc and Dol·PP-$(GlcNAc)_2$ by scaling up (about 10 times) the usual reaction using unlabeled UDP-GlcNAC. The glycolipids (Dol·PP-GlcNAc and Dol·PP-$(GlcNA)_2$) were isolated using the batch DEAE centrifugation procedure described in Methods. These products were then added to the reaction mixture in place of Dol·P. If tunicamycin inhibits the first GlcNAC addition to Dol·P, then only one labeled product, the Dol·P-$(GlcNAc)_2$, should be observed. This is indeed what happened, as can be seen in Figure 5. Channel 1 is the normal reaction using Dol·P only as the acceptor; channel 2 is the same reaction with 0.06 μM tunicamycin added. Tunicamycin effectively inhibited the transfer of GlcNAc to Dol·P. Channel 3 is the reaction run with unlabeled Dol·PP-GlcNAc and Dol·PP-$(GlcNAc)_2$. As expected, two radioactive areas corresponding to Dol·PP-GlcNAc and Dol·PP-$(GlcNAc)_2$ were seen in channel 3. When tunicamycin (0.06 μM) was present in the reaction, only the areas corresponding to Dol·PP-$(GlcNAc)_2$ was observed (channel 4), indicating that the second GlcNAc addition could proceed but not the first.

GDP-Man Effects

Because several reports [5,21,22] had indicated that GDP-Man was necessary for maximal synthesis of oligosaccharide lipids and our initial experiments showed no effect on the production of oligosaccharide lipids by GDP-Man, we investigated the effects of GDP-Man on all three products. Also, other reports [21] indicated that GDP-Man and Mn^{2+} were necessary for optimal oligosaccharide lipid formation. When GDP-Man and Mn^{2+} were added to the reactions, glycolipid synthesis was stimulated but oligosaccharide lipids and glycoprotein synthesis were inhibited by concentrations $\geqslant$ 50 μM (Table 4). Our investigations showed that 2.5 mM Mn^{2+} optimally stim-

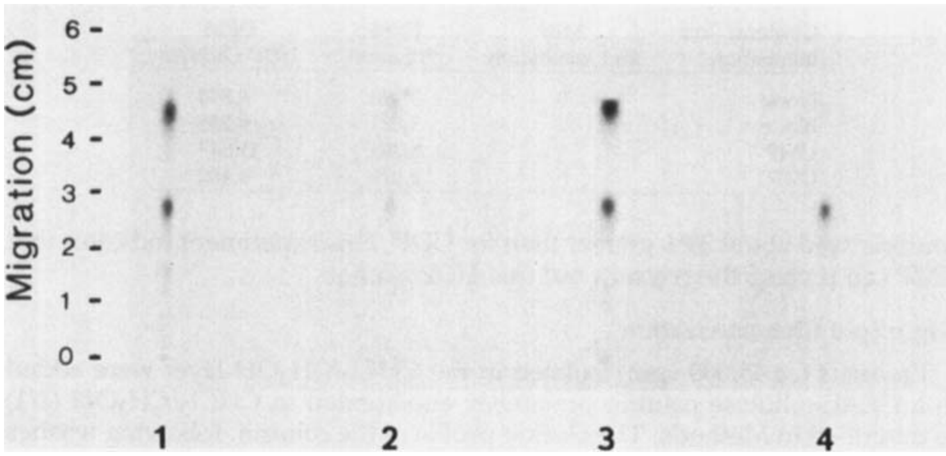

Fig. 5. Autoradiogram of a TLC analysis of the effects of tunicamaycin on the formation of Dol·PP-GlcNAc and Dol·PP-(GlcNAc)$_2$. (1) Control with Dol·P as lipid acceptor; (2) same as (1) but with 50 ng/ml tunicamycin; (3) control using Dol·PP-GlcNAc as the lipid acceptor; (4) same as (3) but with 50 ng/ml tunicamycin. Absence of Dol·PP-GlcNAc in (4) indicates it has been converted to Dol·PP-(GlcNAc)$_2$ and that tunicamycin inhibits the transfer of GlcNAc-1-P from UDP-GlcNAc to Dol·P.

TABLE 4. Effects of GDP-Man on N-Acetylglucosaminyl Transferase

	DPM		
	GL[a]	OL[a]	GP[a]
Control	1,264 ± 112	2,064 ± 123	4,226 ± 480
2.5 μM GDP-Man	1,680 ± 130	2,114 ± 298	2,396 ± 192
50 μM GDP-Man	2,037 ± 982	1,000 ± 150	1,126 ± 150

[a]GL = glycolipids; OL = oligosaccharide lipids; GP = glycoproteins.

ulated oligosaccharide lipid synthesis. The replacement of Mg^{2+} with 2.5 mM Mn^{2+} in the usual reactions stimulated oligosaccharide and glycoprotein synthesis by 50%, but did not stimulate glycolipid synthesis as much as that achieved with Mg^{2+}.

Reversal of N-Acetylglucosaminyl Lipid Formation

The data in Table 3 showing inhibition of GlcNAc transferases by UMP and UDP could possibly be explained by a reversal of the GlcNAc transferase reaction. Table 5 contains the results of an experiment to test for the reversal of this reaction by UMP and UDP. The 0-min control and 5-min control contained approximately the same amount of glycolipid (Dol·PP-GlcNAc and Dol·PP-(GlcNAc)$_2$). In contrast, reaction with UMP contained about 40% of the glycolipids found in the controls, and reaction with UDP about 80%. The amount of radioactivity in UDP-GlcNAc was roughly the same for the 0-min, 5-min control, and UDP samples. The amount of radioactivity in UDP-GlcNAc for the UMP sample was 28–33% greater than for either of the

TABLE 5. Reversal of Glycolipid Accumulation by UMP

Contents 2nd incubation	Min 2nd incubation	DPM glycolipid	DPM UDP-GlcNAc
None	0	3,198	8,894
None	5	3,021	9,235
UMP	5	1,305	11,847
UDP	5	2,371	8,482

controls, and about 39% greater than for UDP. This experiment indicates that UMP can reverse the reaction but that UDP cannot.

Glycolipid Characterization

Products (ca 15,000 cpm) isolated in the $CHCl_3/CH_3OH$ layer were added to a DEAE-cellulose column previously equilibrated in $CHCl_3/CH_3OH$ (2/1) as described in Methods. The elution profile of the column, following washes with 300 ml each of $CHCl_3/CH_3OH$ (2/1), CH_3OH, and 0.1 M ammonium acetate in CH_3OH, is shown in Figure 7. Most of the radiolabeled product (55–60% of the total radioactivity) did not bind to the DEAE-cellulose and eluted in the $CHCl_3/CH_3OH$ wash. This indicates that a large portion of the radioactivity was incorporated into neutral lipid products. Very little radioactivity eluted during the CH_3OH wash. However, when the 0.1 M ammonium acetate in CH_3OH was added to the column, a large radioactive peak constituting about 37% of the original radioactivity eluted. The DEAE-binding characteristic of the second peak is indicative of acidic, phosphate-containing glycolipids.

Radioactive fractions (second peak, Fig. 6) eluting after application of 0.1 M ammonium acetate were pooled. The pooled sample was dried; the residue was dissolved in $CHCl_3/CH_3OH$ (2/1) and washed with H_20 (see Methods) to remove the ammonium acetate. The washed sample was then taken to dryness, redissolved in a minimum amount of $CHCl_3/CH_3OH$ (2/1), and spotted onto silica gel TLC plates. After development in solvent system A, the plates were dried and subjected to autoradiography. Two radioactive areas per channel were observed (Fig. 7)—a slow-moving areas with an R_f of 0.15, and a fast-moving area with an R_f of 0.21. The radioactive areas were scraped from the plates and the samples eluted with $CHCl_3/CH_3OH$ (2/1). The samples were then dried and subjected to mild acid hydrolysis (0.01 N HCl, 100°C, 15 min). After extraction from the hydrolysate, the sample was chromatographed on Whatman No. 1 paper in solvent system D, along with standard GlcNAc and $(GlcNAc)_2$. Radioactivity recovered by hydrolysis of the material from the fast-moving TLC spot migrated in the same area as GlcNAc. The hydrolysis product of the slow-moving TLC spot migrated in the same area as $(GlcNAc)_2$. These results indicate that the slow-moving and fast-moving spots on TLC are, respectively, Dol·PP-$(GlcNAc)_2$ and Dol·PP-GlcNAc.

Characterization of the Oligosaccharide Lipids and Glycoprotein

Sufficient amounts of oligosaccharide lipids (10/10/3, $CHCl_3/CH_3OH/H_2O$-soluble lipids) were obtained by pooling these fractions from several reac-

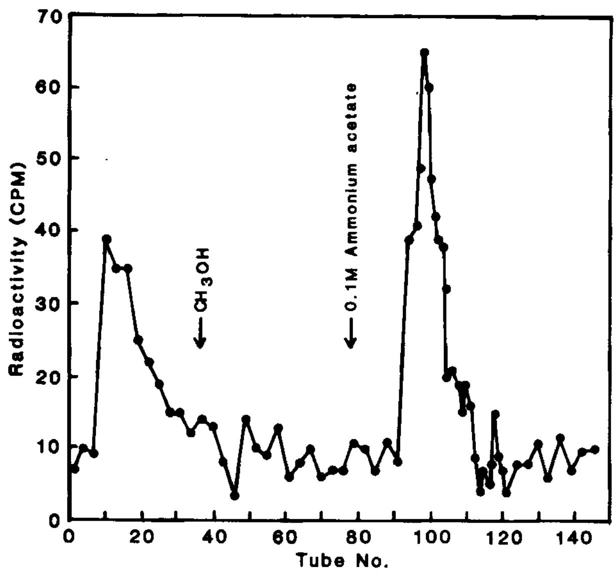

Fig. 6. Column chromatography of the glycolipid fraction on DEAE-cellulose. Procedure as described in Methods.

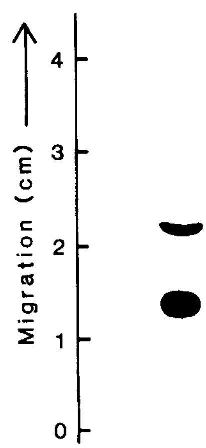

Fig. 7. Autoradiogram of a TLC analysis of the material eluted from the DEAE-cellulose column by 0.1 M ammonium acetate. The silica gel G TLC plate was developed in solvent system A.

tions. When aliquots of these materials were applied to paper chromatograms and run in H_2O, most of the radioactivity stayed at the origin.

Upon mild acid hydrolysis (0.01 N HCl 100°C, 15 min) of the oligosaccharide lipid, the radioactivity migrated near the solvent front of the paper chromatogram. These results are indicative of acid-labile, lipid-linked oligosaccharides.

The glycoproteins were subjected to proteolytic digestion with protease, as described in Methods. Proteolytic digestion released 92–95% of the radioactivity into the soluble fractions, indicating that this fraction was, in fact, glycoprotein.

Attempts were made to solubilize glycoprotein using 1% sodium dodecyl sulfate. Most of the protein (66 ± 4%) was solubilized in 30 min at room temperature. This suggested that the fraction was composed of lipo- or hydrophobic proteins.

Kinetic Constants for Formation of Dol·PP-GlcNAc and Dol·PP-$(GlcNAc)_2$

Apparent kinetic constants were calculated by treating Dol·PP-GlcNAc and Dol·$PP(GlcNAc)_2$ as a single product. Typical Lineweaver-Burk plots are shown in Figure 8 A and B for the collective formation of Dol·PP-GlcNAc and Dol·PP-$(GlcNAc)_2$. The apparent K_m and V_{max} for UDP-GlcNAc was 1.55 ± 0.47 μM and 0.66 ± 0.21 pmol·min^{-1}·mg^{-1} protein, respectively.

Experiments in which Dol·P concentrations were varied whereas UDP-GlcNAc concentrations were held constant gave apparent K_m and V_{max} of 2.08 ± 0.85 μM and 0.13 ± 0.06 pmol·min^{-1}·mg^{-1} protein, respectively.

DISCUSSION

This study involves several different enzyme systems, all of which are capable of transferring GlcNAc units to lipid acceptors or proteins. It is

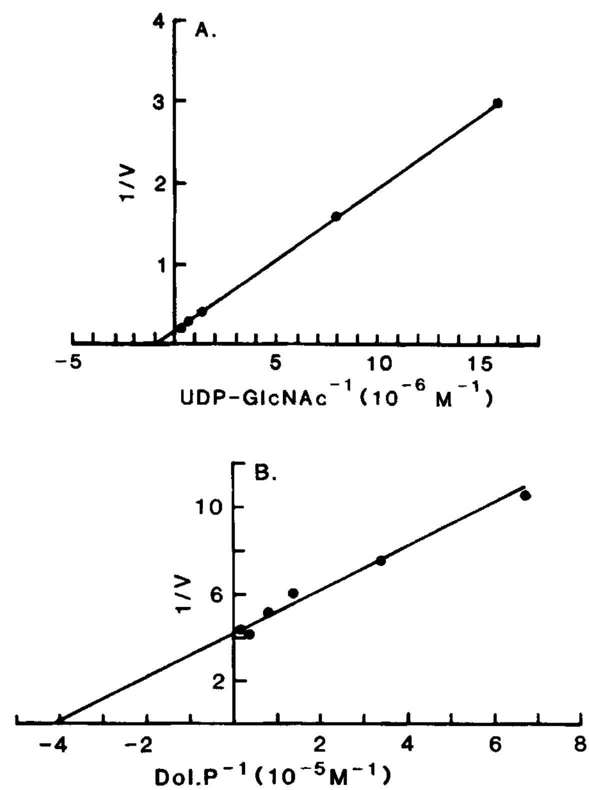

Fig. 8. Typical Lineweaver-Burk plots for the formation of glycolipids while (A) holding Dol·P constant and varying UDP-GlcNAc, and (B) holding UDP-GlcNAc constant and varying Dol·P. V = 10^{-14} mol GlcNAc incorporated·min^{-1}·mg^{-1} protein.

difficult in a study such as this to set up conditions optimal for all of the enzymes involved in the ultimate transfer of large oligosaccharide units to proteins. We therefore tried to optimize conditions for the formation of glycolipids.

Initially, we performed experiments to determine the subcellular distribution of N-acetylglucosaminyl transferases in 0-day pupae (white prepupae) of the stable fly (Table 1). In most cases, the bulk of the activity was associated with the 1,000*g* pellets. However, these fractions had lower specific activities (dpm in glycolipid, oligosaccharide lipid, or glycoprotein/mg protein). The other two fractions with significant activity for glycolipid formation were the 10,000*g* and 100,000*g* pellet fractions. The 100,000*g* pellet fraction had a 44% higher specific activity for glycolipid formation than that of the 10,000*g* fraction. This was to be expected, as most reports on glycosyl transferases utilize microsomal enzyme sources [1,3–5,14,16]. Specific activities for oligosaccharide lipid and glycoprotein formation in the 100,000*g* pellet fraction were low (only 25% and 8%, respectively) compared to those of the 10,000*g* pellet fraction. In this instance, we compromised on glycolipid formation activity to enhance the activities of oligosaccharide lipid and glycoprotein formation by deciding to use the 10,000*g* pellet fraction for our enzyme source.

Investigations of the effects of ions and pH on N-acetylglucosaminyl transferase activity indicate that different enzymes are involved in the various product formations. For example, glycolipid formation was optimal with 2.5 mM Mg^{2+}, but oligosaccharide lipid and glycoprotein formation was unaffected in the former and inhibited in the latter case. The effects of pH further demonstrate that several enzymes are involved with the formation of the different products. Glycolipid, oligosaccharide lipid, and glycoprotein production was optimal at distinctly different pH's.

Inclusion of Dol·P in the reaction mixture more than doubled the amount of glycolipid formed. Similar results have been reported for the stimulation of carbohydrate incorporation from sugar nucleotides into lipid fraction under similar conditions [3]. Large amounts of Dol·P inhibited the formation of oligosaccharide lipids and glycoproteins, but lower amounts had no effect. The observed inhibition may be the result of contaminants in the Dol·P preparations.

N-Acetylglucosaminyl transferase products were characterized by several methods. The glycolipid product appeared to comprise Dol·PP-GlcNAc and Dol·PP-$(GlcNAc)_2$. It bound to DEAE-cellulose and was eluted after addition of 0.1 M ammonium acetate to the column, indicating that the product was strongly acidic. The material eluted with ammonium acetate yielded two radioactive areas when subjected to TLC analysis in a neutral solvent system. Isolation of the radioactive areas, and HCl hydrolysis and subsequent paper chromatography of the H_2O-soluble hydrolysis products, indicated that sugars were GlcNAc and $(GlcNAc)_2$.

The remaining two products, oligosaccharide lipids and glycoproteins, were characterized as acid-labile, lipid-linked oligosaccharides and proteins, respectively. These general characterizations were based on the paper chromatography migration properties of the native oligosaccharide lipids and their hydrolysates in H_2O. Protease and detergent treatment of the glycoprotein fractions indicated that they were proteins.

The profile of N-acetylglucosaminyl transferase activity for the formation of glycolipids during the pupal instar of *S. calcitrans* is surprisingly similar to that of chitin synthase (Fig. 4) [8]. During the first 24 hr of the pupal instar of *S. calcitrans* there is a high rate of membrane synthesis, because imaginal cells are replacing larval cells. Previously, we reported that there is a high rate of chitin synthesis during the first 24 hr and again between 72 and 96 hr [8]. The initial burst of chitin synthesis was associated with the formation of the ecdysial membrane, and the latter with the production of imaginal cuticle. The fact that glycosyl transferase and chitin synthase activities parallel each other may be more than coincidence. Horst [11] has shown that lipid-linked chitin oligosaccharides are formed by brine shrimp microsomes, and suggests that chitin biosynthesis may involve the participation of dolichol oligosaccharide intermediates. Such intermediates may act as primers or play another similar role in the initiation of chitin synthesis in arthropod systems. Support for this idea came from the work of Quesada-Allue [12], who reported that the antibiotic tunicamycin inhibits chitin synthesis in insects. The known action of tunicamycin is to inhibit the transfer of GlcNAc-1-P from UDP-GlcNAc to Dol·P to form Dol·PP-GlcNAc. This mechanism of inhibition has been confirmed here for the first time in insect tissues. It is possible that tunicamycin inhibits the formation of oligosaccharide dolichol intermediates and glycoprotein by preventing the production of Dol·PP-GlcNAc. Thus, chitin synthesis would be inhibited if the intermediates are involved in that process. Another possible explanation for tunicamycin inhibition is that either the synthesis of the chitin polymer or the protein cross linking is N-linked, or both. It is also possible that tunicamycin acts as a competitive inhibitor of chitin synthase as it does in *Neurospora crassa* [26], although this antibiotic has been reported not to have an effect on insect chitin synthesis [27,28].

A recent report [29] suggests that Dol·P-Man may act as an allosteric activator in the formation of GlcNAc-lipids. Thus, Dol·P-Man may play a key role in the regulation of glycoprotein biosynthesis. This finding could in part explain our data on the effects of GDP-Man on GlcNAc-lipid formation. Dol·P-Man could be formed during those reactions that contained GDP-Man, and activate the formation of Dol·PP-GlcNAc and Dol·PP-$(GlcNAc)_2$.

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